application

Agilent Technologies
Innovating the HP Way

Gas Chromatography / Mass Spectrometry | 5973 MSD

Application of Electron Capture Negative Chemical Ionization for the detection of a "Date Rape" Drug

Adam Negrusz, Ph. D.[a], Christine Moore, Ph. D.[b], Harry Prest, Ph. D.[c]

[a] Assistant Professor of Forensic Science, Department of Pharmaceutics and Pharmacodynamics College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612

[b] Director, United States Drug Testing Laboratories, Inc., 1700 South Mount Prospect Road, Des Plaines, IL 60018

[c] Senior Applications Chemist, Agilent Technologies Company, California Analytical Division, 1601 California Avenue, Palo Alto, CA 94304

Introduction

Flunitrazepam is a benzodiazepine marketed by Hoffman-La Roche under the brand name of Rohypnol®. Although the drug is available by prescription as a sleeping pill in Europe and Latin America, there is no legal use of this drug in the United States. Nonetheless, abuse of Rohypnol® has been increasing throughout the southern and southwestern US. Of particular concern is the relatively low street price (USD$ 5) and high potency of Rohypnol®; it is about 10 times more potent than Valium, the most familiar benzodiazepine. Flunitrazepam is commonly abused in conjunction with other drugs especially alcohol. Those who use Rohypnol® and alcohol together frequently experience "blackouts" that last 8 to 24 hours after ingestion. This effect has led to use of flunitrazepam in drug facilitated sexual assault and is known as a "Date Rape Drug." For these reasons, the U.S. Department of Justice Drug Enforcement Agency has classified flunitrazepam as a Schedule I drug, which prohibits possession or trafficking in the U.S. without special exemption.

Analyzing hair is becoming a widely-applied approach to surveying for drugs of abuse. Hair offers several advantages over urine or blood testing. For example, the collection of hair samples is simpler and less invasive than the collection of urine or blood samples and issues of adulteration are minimal with hair. Hair tends to provide a longer documentation period of drug use, of the order of several months, because many compounds tend to remain very stable in hair. (An analysis of hair from 2000 year old Chilean mummies was able to detect the presence of the major metabolite of cocaine.) This is especially important with respect to Rohypnol® because victims of the drug's use may not immediately be aware they have been violated. On the other hand, the difficulty with hair analysis is that the matrix is fairly recalcitrant to digest, and concentrations tend to be very low. As a result, sensitivity must be in the picogram per milligram range.

Metabolism of flunitrazepam produces the 7-aminoflunitrazepam metabolite via reduction of the nitro-group (Figure 1) as one of the three major metabolites (the others being norflunitrazepam and 7-acetamidoflunitrazepam). Since these compounds, like other benzodiazepines, contain electophilic groups such as halogens, nitrogen, and aromatic rings, they are good candidates for detection using electron capture negative ionization (ECNI).

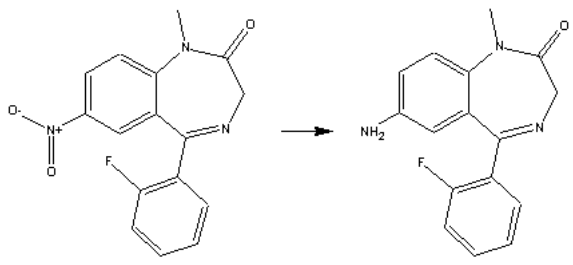

Figure 1. Flunitrazepam [5-(2-fluorophenyl)-1,3 dihydro-1-methyl-7-nitro-1,4-benzodiazepine-2-one] (left) and the analyzed metabolite, 7-aminoflunitrazepam [1-methyl-5(-2-fluorophenyl)-7-amino-1,4-benzodiazepin-2-one] (right)

Experimental

Sample Preparation

A 50-mg hair sample was pulverized using steel balls then transferred to 3 ml of methanol. Flunitrazepam-d_7 and 7-aminoflunitrazepam-d_7 were added as recovery surrogates at 100 pg/mg and 20 pg/mg, respectively, before one hour of sonication. The methanol was then decanted off and 3 ml of 0.1 N HCl is added to the hair and the mixture incubated at 55°C overnight. The supernate was then separated off by centrifugation and combined with the methanol, 1 ml of 1.93 M glacial acetic acid and 9 ml of deionized water. The sample was then added to a pre-conditioned mixed-mode solid-phase extraction column. After drying, the column was eluted with a mixture of dichloromethane, isopropanol, and ammonium hydroxide (78:20:2 v/v/v). The eluate was evaporated under dry nitrogen. The residue was redissolved in 50 µl of ethyl acetate and transferred to an autosampler vial then evaporated to dryness. Heptafluorobutyric acid anhydride (HFBA) was added, the sample incubated at 60°C for 30 minutes and then evaporated to remove excess derivatization reagent. The sample was reconstituted in 25 µl of ethyl acetate. The flunitrazepam was analyzed underivatized and the 7-aminoflunitrazepam was derivatized by the heptafluorobutyric acid anhydride (HFBA).

Instrumental Parameters

The Agilent Technologies 6980/5973 GC/MSD with CI option was operated in the electron capture negative ionization selected ion monitoring mode (SIM) with methane buffer gas. Flunitrazepam was monitored at m/z 313, the 7-aminoflunitrazepam metabolite at m/z 459, and the hepta-deuterated parent and metabolite surrogates at 320 and 466 m/z, respectively.

A more detailed discussion of the extraction and instrumental method parameters will be published shortly.[1]

Results

Figure 2 shows the intense response in electron capture negative ionization SIM of small amounts of flunitrazepam and metabolite extracted from hair on the 5973 MSD. Notice that the background is relatively free of interferences despite the complicated nature of the hair matrix.

Figure 3 and Figure 4 show the linearity of the ECNI response ratio for the parent and metabolite relative to their corresponding surrogates versus the hair concentration over two orders of magnitude. The additional fluorines on the derivatized 7-aminoflunitrazepam greatly enhances the ECNI response over that of the parent.

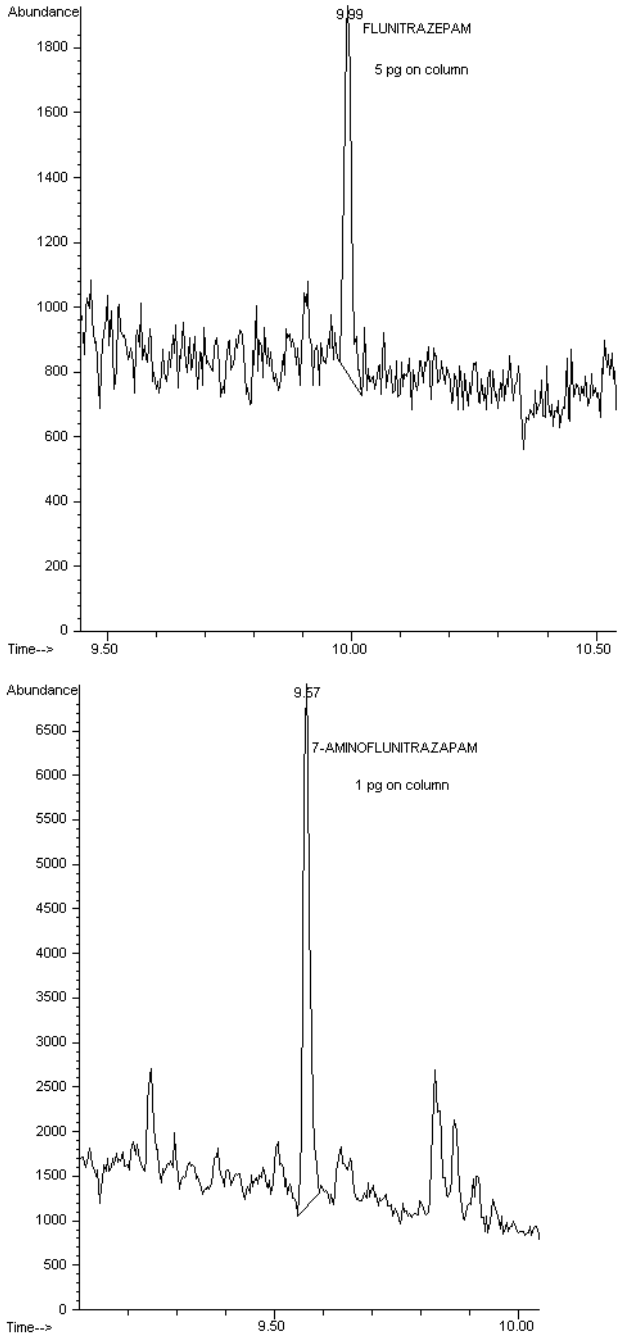

Figure 2. ECNI-SIM chromatograms of 1 pg injected of the derivatized 7-aminoflunitrazepam (lower panel), and 5 pg injected of flunitrazepam (upper panel), both extracted from hair

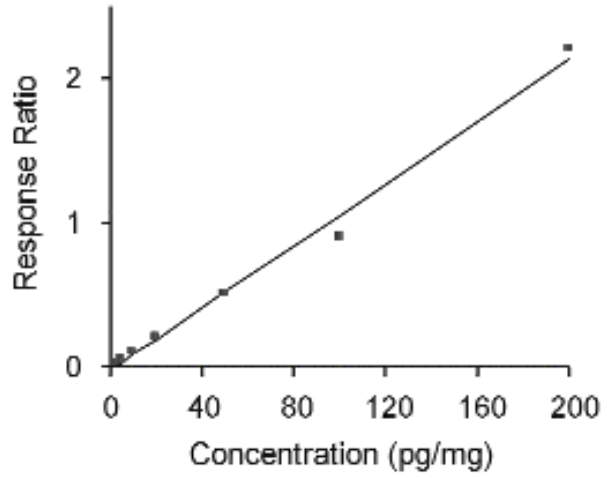

Figure 3. Relative response ratio versus concentration in hair for flunitrazepam from 2.5 to 200 pg/mg, r^2 = 0.996

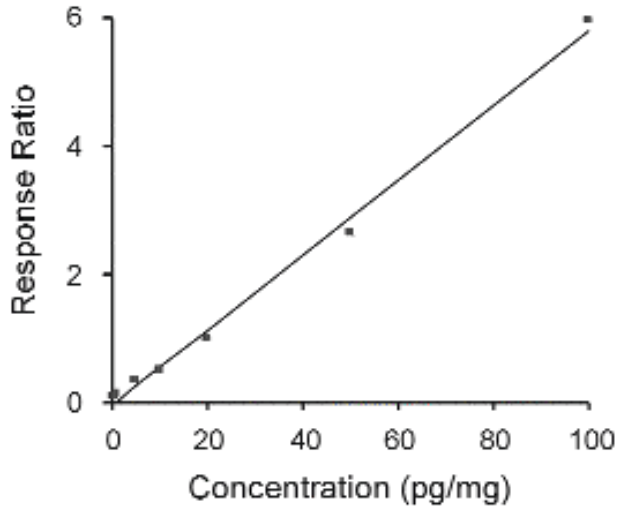

Figure 4. Relative response ratio versus concentration in hair for 7-aminoflunitrazepam from 500 fg/mg to 100 pg/mg, r^2 = 0.998

A study of the intraday and interday accuracy and precision of the method shows very good reliability with high relative accuracy and high relative precision (Table 1 and 2).

Application of the technique to a postmortem hair sample taken from a 70-year old man who died from an overdose indicated parent compound in the hair at a concentration just below the quantitation limit, but a very high concentration of the metabolite (Figure 5). The results are interesting for two reasons: (1) only 9 mg of hair was available, which is 5 times less than is typically analyzed, and (2) the hair was gray. Gray hair lacks melanin which has been shown to be important in the retention of basic drugs in hair.

A low detection limit for the metabolite is also obtained in urine extracts using ECNI SIM. Figure 6 shows results for 7-aminoflunitrazepam in urine at 10 pg/ml which is near the limit of quantitation of the technique. The literature cites methods that have limits of detection using electron impact mass spectrometry with SIM of approximately 10 ng/ml.[2] This improvement of 1000 fold in sensitivity greatly extends the period during which Rohypnol® can be detected, an important advantage in cases of drug-facilitated sexual assault where there can be substantial delays in reporting the crime and because of the relatively low dose that is usually administered.

Table 1. Accuracy and Precision of Flunitrazepam Hair Preparations

Target concentration (pg/mg)	15	80
Intraday study (number of replicates)	N = 4	N = 4
Mean measured concentration (std. dev.)	15.86 (0.75)	71.12 (5.82)
Coefficient of variation as percent	4.73 %	8.18 %
Relative accuracy as percent	5.73 %	–11.10 %
Interday study (number of replicates)	N = 13	N = 13
Mean measured concentration (std. dev.)	14.58 (1.31)	70.40 (4.82)
Coefficient of variation as percent	8.98 %	6.85 %
Relative accuracy as percent	–2.80 %	–12.00 %

Table 2. Accuracy and Precision of 7-Aminoflunitrazepam Hair Preparations

Target concentration (pg/mg)	3	40
Intraday study (number of replicates)	N = 4	N = 4
Mean measured concentration (std. dev.)	2.81 (0.26)	36.31 (3.72)
Coefficient of variation as percent	9.25 %	10.25 %
Relative accuracy as percent	–6.33 %	–9.23 %
Interday study (number of replicates)	N = 14	N = 14
Mean measured concentration (std. dev.)	2.93 (0.28)	38.21 (3.39)
Coefficient of variation as percent	9.56 %	8.87 %
Relative accuracy as percent	–2.33 %	–4.48 %

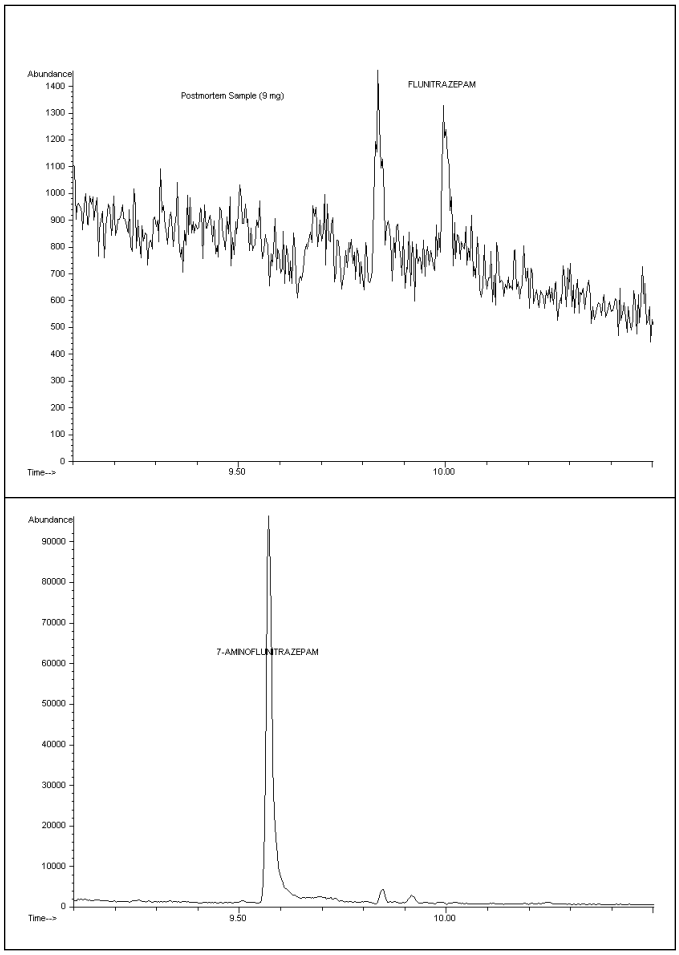

Figure 5. Flunitrazepam (upper panel) and derivatized metabolite (lower panel) in 9 mg postmortem hair sample

References

1. Negrusz, A., et al., *Highly sensitive micro-plate enzyme immunoassay screening and NCI-GC-MS confirmation of flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair.* Journal of Analytical Toxicology, accepted.

2. Lin, Z. and O. Beck, *Procedure for verification of flunitrazepam and nitrazepam intake by gas chromatographic-mass spectrometric analysis of urine.* Journal of Pharmaceutical and Biomedical Analysis, 1995. **13**: p. 719–722.

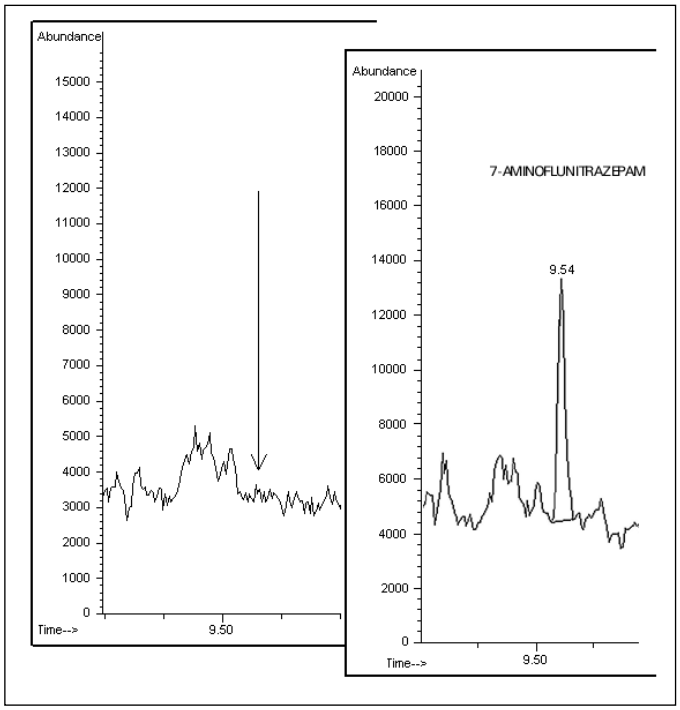

Figure 6. ECNI-SIM chromatograms of urine blank (left panel) and 7-aminoflunitrazepam at 10 pg/ml in urine (right panel)

Acknowledgements

The research performed by Dr. Negrusz and Dr. Moore was partially supported under Award No. 98-LB-VX-K020 from the Office of Justice Programs, National Institute of Justice, Department of Justice. Points of views in this paper are those of the authors and not necessarily represent the official position of the U.S. Department of Justice.

Agilent Technologies shall not be liable for errors contained herein or for incidental or consequential damages in connection with the furnishing, performance or use of this material.

Information, descriptions and specifications in this publication are subject to change without notice.

**Copyright © 1999
Agilent Technologies Company
All rights reserved. Reproduction and adaptation is prohibited.**

**Printed in the U.S.A. December 1999
(23) 5968-4364E**